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## Clinical Evaluation of M30 and M65 ELISA Cell Death Assays as Circulating Biomarkers in a Drug-Sensitive Tumor, Testicular Cancer<sup>1</sup>

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### Abstract

Circulating full-length and caspase-cleaved cytokeratin 18 (CK18) are considered biomarkers of chemotherapy-induced cell death measured using a combination of the M30 and M65 ELISAs. M30 measures caspase-cleaved CK18 produced during apoptosis and M65 measures the levels of both caspase-cleaved and intact CK18, the latter of which is released from cells undergoing necrosis. Previous studies have highlighted their potential as prognostic, predictive, and pharmacological tools in the treatment of cancer. Disseminated testicular germ cell cancer (TC) is a paradigm for a chemosensitive solid malignancy of epithelial origin and has a cure rate of 80% to 90%. We conducted M30/M65 analyses on 34 patients with TC before and during treatment with bleomycin, etoposide, and cisplatin and showed that prechemotherapy serum levels of M65 and M30 antigens are correlated with established TC tumor markers lactate dehydrogenase,  $\alpha$ -fetoprotein, and  $\beta$ -human chorionic gonadotropin, probably reflecting tumor load. Cumulative percentage change of M65 and M30 from baseline to end of study was highest in poor prognosis patients ( $P < .05$ ). Moreover, area under the curve profiles of M65 and M30 during chemotherapy mirrored dynamic profiles for lactate dehydrogenase,  $\alpha$ -fetoprotein, and  $\beta$ -human chorionic gonadotropin. Consequently, M65 and M30 levels appear to reflect chemotherapy-induced changes that correlate with changes in markers routinely used in the clinic for management of patients with TC. This is the first clinical study where M65 and M30 antigen levels correlate with established prognostic markers and provides impetus for their exploration in other epithelial cancers where there is a pressing need for informative circulating biomarkers.

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### Introduction

The development of novel, targeted anticancer therapies coupled with an increased understanding of the molecular processes that occur during cancer progression bring with them an ever more pressing need for the development of clinically robust biomarkers [1,2]. Ideally, these assays may provide information such as optimal patient selection for the design of clinical trials as well as enable real-time evaluation of treatment efficacy and/or toxicity [3,4]. These biomarkers may also facilitate no-go/go decision making that is so crucial during the drug discovery process [5].

Abbreviations:  $\alpha$ FP,  $\alpha$ -fetoprotein; AUC, area under the curve; BEP, bleomycin, etoposide and cisplatin;  $\beta$ HCG,  $\beta$ -human chorionic gonadotropin; ChC, choriocarcinoma; CK18, cytokeratin 18; EC, embryonal carcinoma; IGCCC, International Germ Cell Consensus Classification; IT, immature teratoma; LDH, lactate dehydrogenase; MT, mature teratoma; TC, testicular germ cell cancer; YS, yolk sac tumor

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The M30- and M65-based sandwich ELISAs determine the circulating levels of different forms of the protein cytokeratin 18 (CK18) and are proposed as surrogate biomarkers of drug-induced cancer cell death [6–8]. Cancers of epithelial origin are known to contain relatively large intracellular pools of soluble and insoluble cytokeratins. However, during necrotic and apoptotic cell death, CK18 and other cytokeratins are released into the blood in either their intact or their caspase-cleaved forms where they remain relatively stable in the circulation of patients with cancer [9].

The M30 detection antibody recognizes a neo-epitope mapped to positions 387 to 396 of CK18, so called CK18-Asp<sup>396</sup>, that is only revealed after caspase cleavage of the protein and is postulated as a selective biomarker of apoptosis [10]. The M65 ELISA detects a common epitope present in the full-length protein as well as in the caspase-cleaved fragment [8] and is thus believed to measure, in addition to apoptosis, intact CK18 that is released from cells undergoing necrosis [11]. Both assays have now been validated as “fit for purpose” in the analysis of plasma and serum collected from subjects entered into clinical trials [12–15] and have been extensively applied as pharmacodynamic biomarkers of chemotherapy-induced cell death in a range of different cancer types treated with different chemotherapeutic agents [6,8,16–19].

Testicular germ cell cancer (TC) is the most common malignancy in men between 20 and 35 years old. At the time of diagnosis, up to 50% of patients with TC have disseminated disease and the current standard chemotherapy regimen consists of treatment with a combination of bleomycin, etoposide, and cisplatin (BEP). TC can be considered a paradigm for a chemosensitive solid tumor, as the cure rate for disseminated TC is high (80% to 90%), although about 20% to 30% of the patients diagnosed with disseminated disease will display intrinsic resistance or will acquire resistance to first-line chemotherapy and about 10% will eventually die from TC.

Approximately 90% of the patients with disseminated TC have elevated levels of one or more of the following serum tumor markers: lactate dehydrogenase (LDH),  $\alpha$ -fetoprotein ( $\alpha$ FP), and  $\beta$ -human chorionic gonadotropin ( $\beta$ HCG). Normalization of these tumor markers is the most commonly used criterion for favorable tumor response [20] and levels usually decrease within 5 to 12 days after the start of chemotherapy [21]. When marker levels normalize during or after completion of chemotherapy, patients are considered to have had a complete biochemical tumor response. In the cases where residual disease is present, it will be treated surgically [22,23]. Upon complete remission, regular measurement of serum tumor markers is an essential component of the follow-up of patients with TC [24,25]. In addition, LDH,  $\alpha$ FP, and  $\beta$ HCG are used, in combination with the localization of the primary tumor and the presence of nonpulmonary visceral metastases, for prognosis estimation according to the International Germ Cell Consensus Classification (IGCCC). Initial increase in  $\alpha$ FP levels after start of chemotherapy appears to be associated with unfavorable outcome [26].

It must be noted, however, that initial levels of these serum tumor markers have a limited predictive value for disease outcome in the individual patient. Moreover, up to 10% of patients with disseminated TC do not have elevated tumor markers. Thus, additional serological biomarkers may contribute to a better prediction of response to chemotherapy and disease outcome.

We have conducted an exploratory study where we have investigated changes in serum and plasma concentrations of total CK18 (M65 antigen) and caspase-cleaved CK18 (M30 antigen) in patients

with TC before and serially after treatment with BEP chemotherapy and their association with changes in the clinically used tumor markers LDH,  $\alpha$ FP, and  $\beta$ HCG and treatment outcome.

## Patients and Methods

### *Patients and Collection of Blood Samples for Analysis of CK18 and Caspase-Cleaved CK18*

Blood samples were collected from two groups of patients and analyzed for the acute effects of chemotherapy for disseminated TC. All patients received BEP chemotherapy, consisting of a combination of bleomycin (30 mg on days 2, 8, and 15 of each course), etoposide (100 mg/m<sup>2</sup> on days 1 to 5), and cisplatin (20 mg/m<sup>2</sup> on days 1 to 5) for three or four courses of 3 weeks.

We collected blood samples of 11 consecutive patients with TC participating in a prospective study on acute effects of BEP chemotherapy and treated from May 2006 to November 2006. For this study, both serum and heparin plasma were collected on day 1 (before start of chemotherapy) and heparin plasma for days 2 (24 hours after start), 3 (48 hours after start), 6, 8, and 15 of the first course and days 1, 8, and 15 of the following courses. Samples were stored at  $-20^{\circ}\text{C}$ .

To extend our exploratory analysis, we selected from our serum bank a group of 23 patients with TC treated with BEP chemotherapy at the University Medical Center Groningen, the Netherlands, between January 2004 and May 2006. Selection was based on the availability of serum samples and diversity with respect to the histologic composition of the primary tumor, IGCCC prognosis group, disease outcome, and levels of tumor markers LDH,  $\alpha$ FP, and  $\beta$ HCG. From the stored serum samples (at  $-20^{\circ}\text{C}$ ) we selected samples from before the start of chemotherapy and days 1, 8, and 15 of each chemotherapy course. If no samples were available for days 1, 8, or 15, a sample from 1 day before or after was analyzed.

The analysis of blood samples was approved by the local medical ethical committee and written informed consent was obtained from each participant.

### *Immunohistochemistry for CK18 and Caspase-Cleaved CK18 in Tumor Material*

Paraffin-embedded testicular tumor material was collected for each patient before the start of chemotherapy and analyzed for CK18 and caspase-cleaved CK18. For immunohistochemistry, 3- $\mu\text{m}$  sections were cut and placed on 3-Aminopropyltriethoxysilane-coated slides, deparaffinized and dehydrated. The presence of testicular germ cell tumor components was confirmed by a pathologist using standard hematoxylin-eosin staining. For staining of CK18, endogenous peroxidase was blocked (30 minutes, 0.3% H<sub>2</sub>O<sub>2</sub>); in addition, sections were pretreated with 0.1% protease XXIV for 30 minutes (Sigma-Aldrich, St. Louis, MO) followed by incubation with avidin and, subsequently, biotin blocking solution, both for 15 minutes (Vector Laboratories, Burlingame, CA). For staining of caspase-cleaved CK18, sections were preheated by microwave for 8 minutes at 700 W in citrate buffer (0.1 M, pH 6.0) followed by blocking of endogenous peroxidase activity as described above.

Subsequently, slides were incubated for 1 hour with the primary antibody: CAM5.2 (Becton-Dickinson, San Jose, CA) recognizing both CK18 and CK8, which show a similar tissue distribution, or

M30 antibody (Boehringer Mannheim GmbH, Mannheim, Germany) to detect CK18-Asp<sup>396</sup>. A secondary biotinylated rabbit anti-mouse antibody followed by a tertiary StreptAB-complex/HRP and peroxidase-conjugated rabbit anti-mouse antibody followed by peroxidase-conjugated goat anti-rabbit antibody (antibodies from DAKO, Glostrup, Denmark) were used, respectively. DAB was used as chromagen to visualize peroxidase activity. Counterstaining was performed with hematoxylin. Immunoglobulin class-matched control sera were used as negative controls. Normal appendix tissue served as a positive control for CK18 and CK8, and colon carcinoma tissue for caspase-cleaved CK18.

### Measurement of CK18 and Caspase-Cleaved CK18 in Tumor Material by Immunohistochemistry

Evaluation of staining was performed by a pathologist without knowledge of the clinical data. With respect to CK18/CK8 (recognized by CAM5.2), tumor components showing no positive cells were considered negative (-), whereas tumor components showing stained tumor cells were considered positive (+ in the presence of focal positive staining or + in the presence of diffuse positive staining). For caspase-cleaved CK18, tumor components showing positivity in less than five high-power fields (with a field diameter of 0.55 mm) were considered negative (-). Focal positivity for caspase-cleaved CK18 was scored +, whereas the presence of massive positivity was scored ++. Heterogeneity and pattern of staining regarding each tumor component were also recorded.

### Determination of CK18 (M65 ELISA) and Caspase-Cleaved CK18 (M30 ELISA) in Blood

The M30 apoptosense and M65 ELISA kits were both obtained from PEVIVA AB (Bromma, Sweden), and these assays, previously validated for clinical trial use, were performed under dedicated Good Clinical Laboratory Practice conditions as previously described [12–14]. Background variation for M65 and M30 antigens is considered as  $\pm 30\%$  of the antigen level seen at the start of each treatment cycle as discussed previously [13]. Any peaks or troughs seen in patient antigen levels falling outside this range may be considered a direct result of chemotherapy: either tumor response or toxicity.

### Statistics

Data were analyzed statistically with SPSS software package (SPSS Inc., Chicago, IL). Groups were compared for M65 and M30 by using the Mann-Whitney *U* test, whereas changes in M65 and M30 over time were tested with the paired Wilcoxon signed rank test. The Kruskal-Wallis nonparametric test was performed to test for significant differences among the distributions of the three prognosis groups. Post hoc pairwise comparisons were made with Bonferroni correction when appropriate.

To calculate the cumulative percentage change in M65 and M30 levels over the entire treatment period, values with a 30% threshold from the predose value for each chemotherapy course were judged to be either a “peak” (increase above threshold), a “trough” (decrease below threshold), or “no change” (remaining either above, below, or within the 30% threshold in line with the previous reading) [13]. At each peak and trough, the absolute values of M30 and M65 were recorded and summed over the entire treatment period to give the cumulative percentage change.

To calculate the area under the curve (AUC) values from the measured concentrations at sequential, specific sampling points, the trapezoidal equation was used as follows:

$$\sum_{n=1}^n \left\{ \frac{c_n + c_{n+1}}{2} (t_{n+1} - t_n) \right\}$$

where  $c_n$  and  $t_n$  are the concentration and time point, respectively, for the  $n$ th time point. To determine the extent to which the AUC values of M65, M30, and the standard tumor markers LDH,  $\alpha$ FP, and  $\beta$ HCG are proportional to each other, weekly AUC data for all five markers were normalized between 0 and 1 using minmax normalization [27] so that a direct comparison could be made between these markers in terms of the AUC profile over the entire period. All tests were performed two-sided and *P* values  $< .05$  were considered statistically significant.

## Results

### Patient Characteristics

General patient characteristics are summarized in Table 1. Among the total group of 34 patients there were 4 patients with refractory disease.

### CK18 and Caspase-Cleaved CK18 (M30) in Primary Tumor Material

For each patient, every testicular germ cell tumor component was scored separately for the expression of CK18/CK8 and caspase-cleaved CK18. All nonseminomatous histologic components were positive for CK18/CK8 (all +), with the embryonal carcinoma (EC) component showing most positivity (Figure 1A), followed by the choriocarcinoma (ChC) component. These two components were also most often positive for caspase-cleaved CK18 (EC, 22/22 ++; ChC, 1/6 + and 5/6 ++), with the variable presence of so-called aponecrosis in EC components. Noteworthy, the yolk sac tumor (YS) component was negative for caspase-cleaved CK18 when it had the

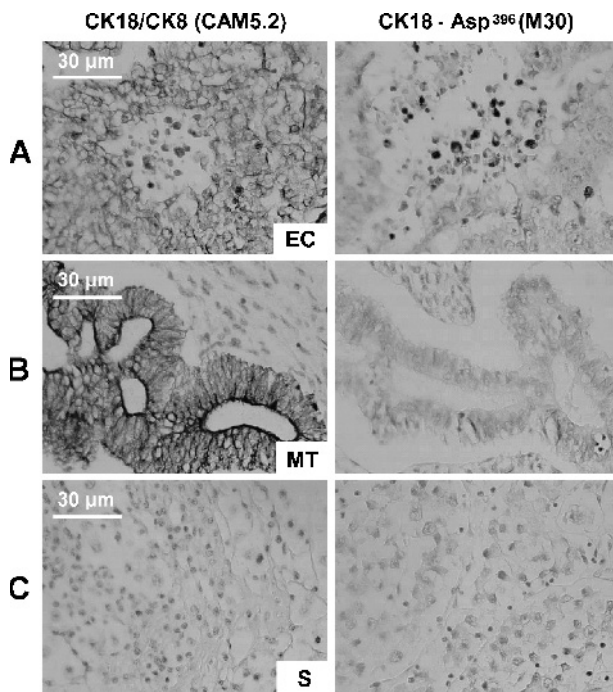
Table 1. General Patient Characteristics.

	Prospective Group	Serum Bank Group
No. of patients	11	23
Age, median (range) (years)	35 (19–46)	31 (19–53)
Histology, <i>N</i> (%)		
Nonseminoma	10 (91)	21 (91)
Seminoma	1 (9)	2 (9)
Stage, <i>N</i> (%) <sup>*</sup>		
II	9 (82)	15 (65)
III	1 (9)	1 (4)
IV	1 (9)	7 (31)
Prognosis group, <i>N</i> (%) <sup>†</sup>		
Good	10 (91)	11 (48)
Intermediate	1 (9)	7 (30)
Poor	0 (0)	5 (22)
Chemotherapy regimen, <i>N</i> (%)		
4 BEP	2 (18)	20 (87)
3 BEP	9 (82)	3 (13)
Response to chemotherapy, <i>N</i> (%)		
Complete biochemical response	4 (36)	7 (30)
Complete biochemical response + surgery	6 (55)	13 (57)
Teratoma	4	8
Necrosis	2	5
Refractory disease	1 (9)	3 (13)

<sup>\*</sup>Royal Marsden classification.

<sup>†</sup>IGCC classification.





**Figure 1.** Expression of CK18/CK8 (CAM5.2) and caspase-cleaved CK18 (M30) in different testicular germ cell tumor components. (A) Embryonal carcinoma (EC): positive for CK18/CK8 and apoptotic cells are positive for caspase-cleaved CK18. (B) Mature teratoma (MT): in general positive for CK18/CK8 and negative for caspase-cleaved CK18. (C) Seminoma (S): negative for both CK18/CK8 and caspase-cleaved CK18.

reticular pattern, whereas it showed positivity for caspase-cleaved CK18 with aponecrosis in the solid pattern, which is histologically more similar to the EC pattern (YS, 3/6 –, 2/6 +, and 1/6 ++). Mature teratoma (MT) and immature teratoma (IT) components showed variable expression of caspase-cleaved CK18 (MT, 11/12 – and 1/12 +; IT, 3/5 – and 2/5 +) (Figure 1B).

The seminoma (S) component was negative for both CK18/CK8 (7/8 – and 1/8 ±) and caspase-cleaved CK18 (8/8 –; Figure 1C) with the exception of one patient who had a mixed germ cell tumor with focal positivity for CK18/CK8 in the S component.

#### *Circulating CK18 (M65) and Caspase-Cleaved CK18 (M30) before Chemotherapy*

Prechemotherapy sera were taken and analyzed for M65 and M30 antigen levels from 23 retrospectively sampled patients; data were grouped according to IGCCC prognosis group guidelines (good, intermediate, or poor). Serum levels of M65 and M30 antigens varied significantly according to prognosis group (Figure 2A). Patients in the poor-prognosis group showed the highest median level of M65 (2456 U/l) followed by the intermediate-prognosis group (642 U/l) and then the good-prognosis group (366 U/l). Median M30 levels follow the same pattern (poor-prognosis group, 648 U/l; intermediate-prognosis group, 219 U/l; and good-prognosis group, 144 U/l).

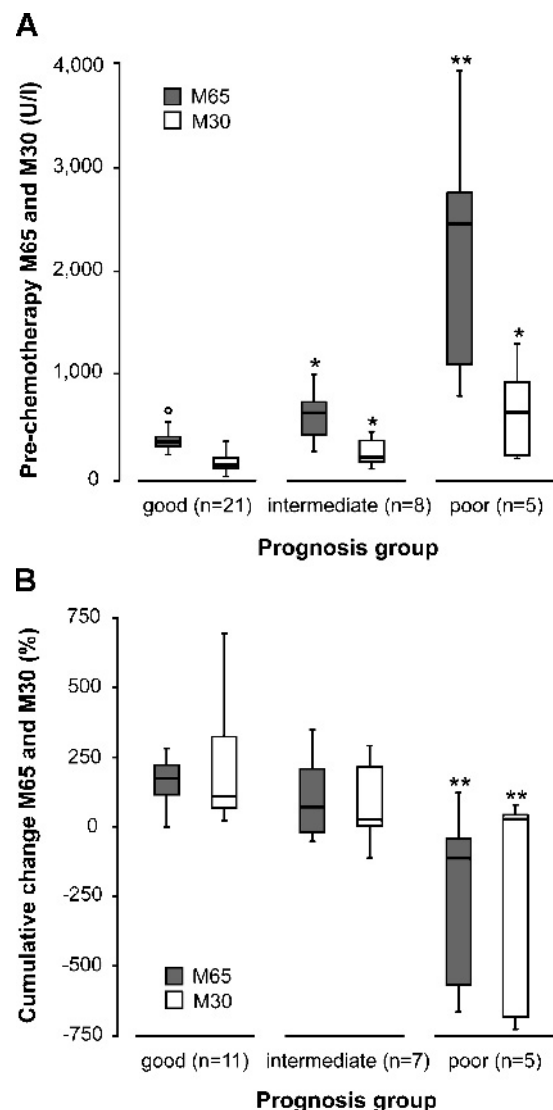
#### *Changes in Circulating CK18 (M65) and Caspase-Cleaved CK18 (M30) during the First Chemotherapy Course*

For the 11 patients participating in the prospective study, data were available at early time points at 24 hours, 48 hours, 5 days, 7 days, 14 days, and 21 days after start of chemotherapy (first chemotherapy

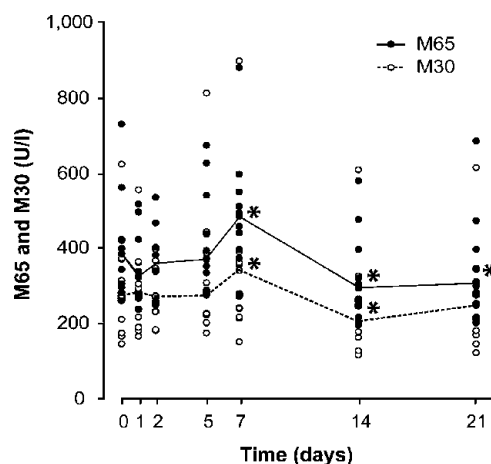
course) (Figure 3). Up to 7 days after start of chemotherapy there was a significant increase in median M65 level (prechemotherapy, 384 U/l, after 7 days 483 U/l;  $P = .010$ ) and median M30 level (prechemotherapy, 267 U/l; after 7 days, 338 U/l;  $P = .026$ ). This peak was followed by a significant decrease in M65 levels compared to baseline values resulting in a median level of 294 U/l at 14 days ( $P = .021$ ) and 307 U/l ( $P = .016$ ) at 21 days after start of chemotherapy. M30 levels were also decreased compared to prechemotherapy levels 14 days after start of treatment (median, 203 U/l;  $P = .008$ ).

#### *Circulating CK18 (M65) and Caspase-Cleaved CK18 (M30) Profiles in Patients from the Three IGCCC Prognosis Groups*

Typical examples of individual patient profiles from each of the three groups are shown in Figure 4. Arrows indicate the start of each



**Figure 2.** (A) Prechemotherapy serum levels of total CK18 (M65) and caspase-cleaved CK18 (M30) according to prognosis group (IGCCC) in all groups of analyzed patients ( $n = 34$ ). (B) Cumulative percentage changes in total CK18 (M65) and caspase-cleaved CK18 (M30) levels over entire treatment period grouped by IGCCC prognosis for samples collected from serum bank ( $n = 23$ ). \*Significant difference from good-prognosis group ( $P < .05$ ). \*\*Significant difference from good and intermediate-prognosis group ( $P < .05$ ).



**Figure 3.** Changes in circulating CK18 (M65) and caspase-cleaved CK18 (M30) in serum of patients with TC ( $n = 11$ ) during the first course of BEP chemotherapy. The median values are connected by a line. \*Median level significant different from baseline ( $P < .05$ ).

cycle of chemotherapy and error bars at each of these points indicate the 30% signal-to-noise ratio; thus, values outside this range are considered significant [13]. Patients in the good and intermediate-prognosis group (Figure 4, A and B, respectively) displayed repeated drug-induced spikes in M30 and M65 levels throughout the course of therapy, whereas M65 and M30 antigen levels for the patients in the poor-prognosis group were initially very high and declined rapidly on the first chemotherapy course with the absence of clear peaks (Figure 4C). The four patients with refractory disease showed a profile similar to that of the 30 patients with a favorable tumor response.

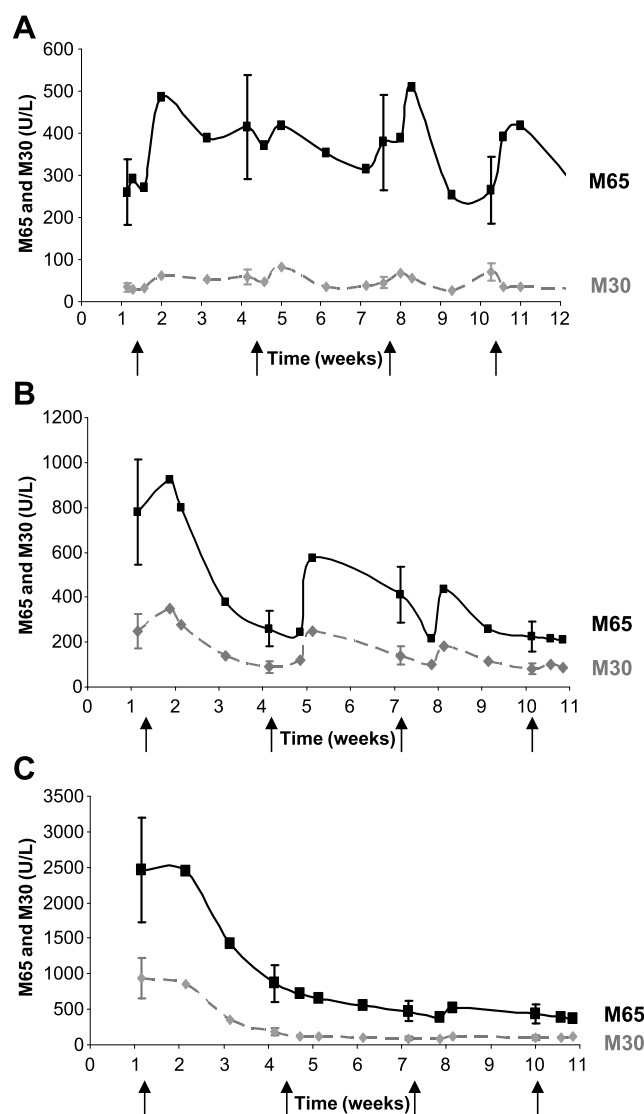
#### Cumulative Changes in Circulating CK18 (M65) and Caspase-Cleaved CK18 (M30) over the Entire Treatment Period

Changes in circulating M65 and M30 were analyzed in the 23 patients for whom samples were available throughout their entire treatment regime. Cumulative percentage changes in M65 (good prognosis, 166.5%; intermediate prognosis, 121.3%; and poor prognosis, -119.4%) and M30 (good prognosis, 90.4%; intermediate prognosis, 69.7%; and poor prognosis, 25.5%) over the entire treatment period differed highly according to IGCCC prognosis group. Patients in the poor-prognosis group showed the largest cumulative decrease in both M65 and M30 (Figure 2B). The median cumulative change in M65 in the poor-prognosis group differed significantly from the good-prognosis group ( $P = .002$ ) and intermediate-prognosis group ( $P = .008$ ), whereas the median cumulative changes in M30 also differed between the poor-prognosis patients and good-prognosis patients ( $P = .011$ ), but not between the poor- and intermediate-prognosis patients.

#### Correlation between Circulating CK18 (M65), Caspase-Cleaved CK18 (M30), and Standard Tumor Markers

Prechemotherapy serum levels of M65 and M30 were strongly positively correlated with circulating levels of the standard TC tumor markers LDH,  $\beta$ HCG and  $\alpha$ FP (Table 2).

AUC analysis of median week-by-week changes in M65 (Figure 5A) showed a significant difference according to IGCCC prognosis group during the first 2 weeks of chemotherapy ( $P < .05$ ) and



**Figure 4.** M30 and M65 profiles in a patient from the three IGCCC-defined prognosis groups: (A) good prognosis, (B) intermediate prognosis, (C) poor prognosis. Serum from patients receiving standard chemotherapy for TC was analyzed for circulating CK18 (M65) and caspase-cleaved CK18 (M30). Arrows indicate the start of each chemotherapy course and error bars indicate 30% signal-to-noise ratio for each treatment cycle.

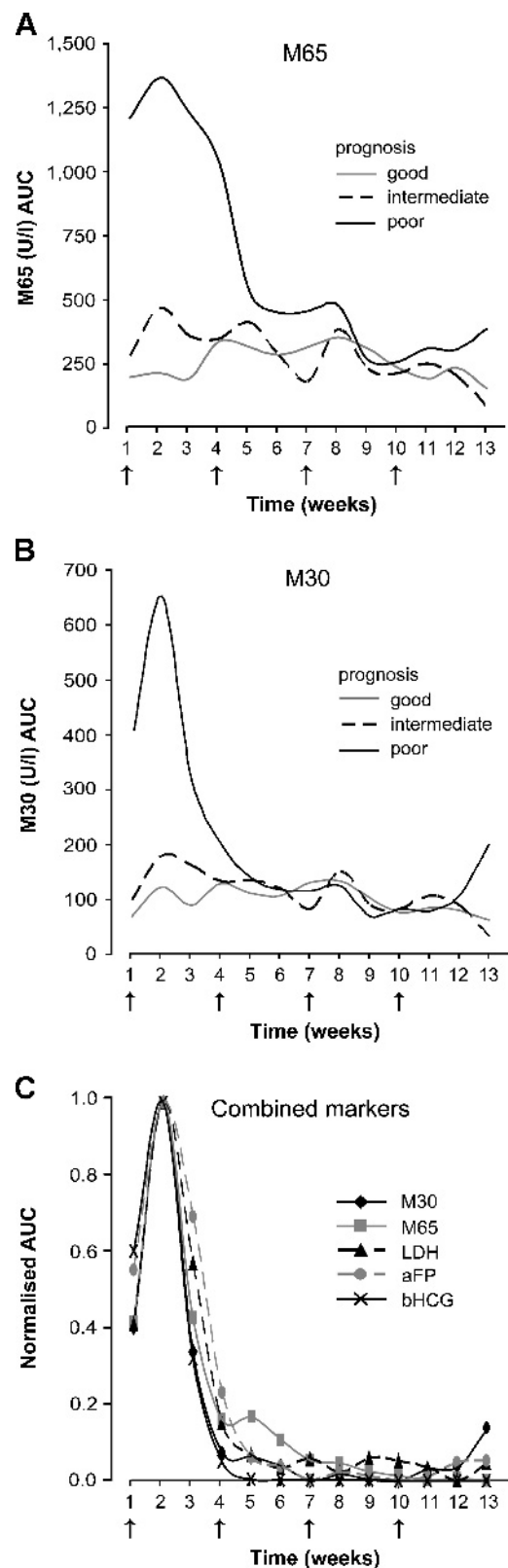
was able to distinguish patients in the poor-prognosis group during subsequent rounds of treatment ( $P < .05$ ). Similarly, M30 levels showed significant variation according to prognosis group during the first 2 weeks of treatment ( $P < .05$ ) (Figure 5B). Of particular

**Table 2.** Correlation between Circulating CK18 (M65), Caspase-Cleaved CK18 (M30) in Serum, and Standard Tumor Markers Before Start of Chemotherapy.

Tumor Marker	Correlation Coefficient (Spearman's Bivariate Correlation Test)			
	M65	P	M30	P
LDH ( $n = 34$ )	<b>0.698</b>	0.000	<b>0.586</b>	0.000
$\alpha$ FP ( $n = 20$ )*	0.129	0.588	<b>0.475</b>	0.034
$\beta$ HCG ( $n = 22$ )*	<b>0.866</b>	0.000	<b>0.864</b>	0.000

Values in bold indicate significance ( $P < .05$ ).

\*Analysis includes patients in whom concerned tumor marker was elevated before start of chemotherapy.



**Figure 5.** AUC data for (A) circulating CK18 (M65) and (B) caspase-cleaved CK18 (M30) according to IGCCC prognosis group. (C) Levels of M65, M30, and standard tumor markers LDH,  $\alpha$ FP, and  $\beta$ HCG combined as normalized AUC data. The arrows indicate the start of each chemotherapy course.

**Table 3.** Correlation between Serum Levels of CK18 (M65), Caspase-Cleaved CK18 (M30) in Serum, and Standard Tumor Markers during Chemotherapy, Using Weekly AUC Data.

Prognosis Group	Tumor Marker	Correlation Coefficient (Spearman's Bivariate Correlation Test)	
		M65	M30
Good ( <i>n</i> = 11)	LDH ( <i>n</i> = 11)	<b>0.804</b>	<b>0.593</b>
	$\alpha$ FP ( <i>n</i> = 5)*	-0.262	0.182
	$\beta$ HCG ( <i>n</i> = 8)*	-0.295	0.092
Intermediate ( <i>n</i> = 7)	LDH ( <i>n</i> = 7)	<b>0.654</b>	<b>0.579</b>
	$\alpha$ FP ( <i>n</i> = 4)*	0.546	0.547
	$\beta$ HCG ( <i>n</i> = 5)*	<b>0.561</b>	<b>0.579</b>
Poor ( <i>n</i> = 5)	LDH ( <i>n</i> = 5)	<b>0.980</b>	<b>0.967</b>
	$\alpha$ FP ( <i>n</i> = 4)*	<b>0.975</b>	<b>0.963</b>
	$\beta$ HCG ( <i>n</i> = 5)*	<b>0.963</b>	<b>0.973</b>

Values in bold indicate significance ( $P < .05$ ). Samples were taken from the retrospective study ( $n = 23$ ).  
\*Analysis includes patients in whom concerned tumor marker was elevated before start of chemotherapy.

note is the finding that AUC analyses of M65 and M30 levels during chemotherapy exhibited profiles that were remarkably similar to those of the standard tumor markers (Figure 5C). Spearman's correlation analysis showed a significant correlation between both M65 and M30 with LDH in all three prognosis groups,  $\beta$ HCG in intermediate and poor-prognosis groups, and  $\alpha$ FP in the poor-prognosis group ( $P < .05$ ; Table 3).

Discussion

The high levels of intact and caspase-3 cleaved CK18 in the circulation of patients with epithelial malignancies have been attributed to intracellular proteins that have been shed into the blood from dying tumor cells [16,18,19]. Similarly, increases in circulating levels of CK18 after chemotherapy have also been reported, and thus this protein is considered to be a serological biomarker of tumor cell death [16,19,28].

The expression of CK18 in nonseminomatous histologic components of TC renders CK18 and its caspase-cleaved fragment CK18-Asp<sup>396</sup> candidate biomarkers for chemotherapy-induced tumor cell death and cell death mode (apoptosis vs. necrosis) in nonseminomatous patients with TC. This exploratory study shows that serum levels of CK18 (M65 antigen) and caspase-cleaved CK18 (M30 antigen) before chemotherapy are associated with prognosis group according to IGCCC classification and are correlated with prechemotherapy levels of tumor markers LDH,  $\alpha$ FP, and  $\beta$ HCG. In addition, changes in M65 and M30 are observed during chemotherapy. These changes consist of an overall decrease combined with peaks during most chemotherapy courses in the good and intermediate-prognosis group. AUC analysis shows that changes in M65 and M30 are strongly correlated with chemotherapy-induced changes in LDH,  $\alpha$ FP, and  $\beta$ HCG.

In our patients, CK18 and caspase-cleaved CK18 levels also vary with different histologic components of germ cell tumors [29,30]. It seems conceivable that the large variation in blood levels of M65 and M30 before chemotherapy is partially caused by the present tumor mass or masses, their histologic composition, and the baseline levels of spontaneous tumor cell death. Moreover, the observed association with IGCCC prognosis group and tumor markers LDH,  $\beta$ HCG, and  $\alpha$ FP suggests that the height of M65 and M30 levels is primarily influenced by tumor load.

Because CK18-Asp<sup>396</sup> is expressed during apoptosis and released during loss of cell membrane integrity with further progression of apoptosis or secondary necrosis, we prospectively analyzed changes in serum M65 and M30 in 11 patients with TC shortly after start of chemotherapy. An increase in M65 and M30 levels was observed



up to 7 days after start of the chemotherapy. In previous studies on the effects of chemotherapy in patients with breast cancer and prostate cancer, increases in M65 and M30 levels were found within 1 to 3 days after start of chemotherapy [17,31]. We observed most significant changes 7 days after start of treatment, which may reflect the cumulative effect of the 5-day dosing scheme of cisplatin and etoposide during BEP chemotherapy.

During the following courses, an overall decrease is observed with the superposition of peaks for both M65 and M30 after start of a new chemotherapy course in good- and intermediate-prognosis patients. Poor-prognosis patients show the most pronounced overall decrease in M65 and M30. The profiles and AUC change of M65 and M30 appear to mirror the profiles of the standard tumor markers both before the start of chemotherapy and throughout treatment. This strong correlation suggests that the overall decrease in M65 and M30 are indicative of treatment response, as they appear to reflect a decrease in tumor load due to chemotherapy-induced tumor cell death. A comparable association has been found between docetaxel-induced increases of M30 and baseline levels of PSA, reflecting tumor volume in patients with prostate cancer [17]. In addition, serum M30 levels in patients with breast cancer correlated with the number of involved organs [18].

In addition, the peaks of M65 and M30 observed after the start of each treatment cycle in good and intermediate-prognosis group patients indicate a drug-induced effect, which may reflect tumor response. The fact that these peaks are not observed in patients with poor prognosis is possibly related to high initial levels of M65 and M30. With this exploratory analysis in a small number of selected patients, it cannot be proven that M65 and M30 peaks are specific enough for chemotherapy-induced tumor cell death. Neither can it be excluded that these peaks (partially) reflect chemotherapy-induced toxicity to normal epithelial tissue. Recently, elevated serum levels of caspase-cleaved CK18 have been found in, for instance, patients with an acute myocardial infarction [32].

The four patients with TC who eventually did not respond to BEP chemotherapy after an initial decline in tumor markers showed patterns of M30 and M65 comparable to responding patients, including chemotherapy-induced peaks. In case these peaks are tumor cell death related, they may represent cell death of chemotherapy-sensitive subpopulations of cells, reflected by initial decrease of tumor markers. However, the number of refractory patients in this study is too small to draw firm conclusions. Consequently, the presence of chemotherapy-induced peaks in M65 and M30 may not exclude future treatment failure. Correspondingly, increases in CK18 have been observed in breast cancer patients with stable disease instead of clinical response to chemotherapy [31].

With regard to the treatment of disseminated TC, the question remains whether determination of M65 and M30 has additional value for monitoring tumor response to chemotherapy. These results suggest that M30 and M65 may reflect drug-induced changes in tumor; however, to have a predictive value for the individual patient, serum M65 and M30 need to show distinct patterns for patients with a favorable disease outcome and for those with a nonfavorable disease outcome. Fortunately, because the cure rate is so high for disseminated TC (80% to 90%) a larger study containing more nonresponding patients is needed to assess whether changes in M65 and M30 are specific enough for tumor cell response and whether prechemotherapy levels and early changes in M65 and M30 are predictive for disease outcome in testicular cancer patients treated with chemotherapy.

This study is the first example in a clinical setting where circulating levels of M30 and M65 antigen correlate with internationally recognized circulating biomarkers that are routinely and successfully used as prognostic indicators and for monitoring treatment response in TC. The correlation between M65/M30 levels and IGCC prognostic group and their overall agreement with LDH,  $\alpha$ FP, and  $\beta$ HCG levels suggest that M65/M30 may also have a prognostic value in patients with TC. This concordance of M30 and M65 with these prognostic markers of TC adds to the momentum for future exploration of M30 and M65 in other epithelial cancers where informative circulating biomarkers are needed [16,17,19,31].

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